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Flow cytometric quantification of viruses in activated sludge

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ABSTRACT

Viruses may play a critical role in the microbial dynamics of activated sludge systems; however the difficulty of their quantification makes long term and large scale studies costly, timely and challenging. Thus a flow cytometric protocol was optimised and employed to determine virus abundance in activated sludge samples. The best flow cytometry signature and highest virus count was obtained by separating the indigenous floc-associated viruses using Tween 80 and sodium pyrophosphate, diluting the sample with Tris–EDTA and staining with SYBR Green II. Using the optimised protocol viral concentrations from 25 activated sludge plants were determined, with average concentrations of 2.35×10^9 mL^{-1} observed. Direct counts by transmission electron microscopy were highly correlated with flow cytometric counts ($p = <0.05$ and $r^2 = 0.77$), with concentrations from both quantification methods comparable at the order of magnitude level. The high counting efficiency, ease of preparation and rapidity and reproducibility of analysis makes flow cytometric quantification of viruses in activated sludge ideal for routine investigation and thus invaluable in unravelling the complexity of phage host interactions in such systems.

1. Introduction

Bacteria are an integral part of activated sludge (AS) processes; dozens, perhaps hundreds, of different species play key roles in nutrient removal and the transformation and mineralisation of organic matter (Shapiro and Kushmaro, 2011). Consequently factors controlling bacterial abundance, diversity and activity are central to understanding, developing and predicting the behaviour of such processes. Among these factors, top down control through viral lysis could have an important role. Bacteriophages (viruses that infect bacteria) are the most abundant and diverse biological entities on earth, typically in the order of 10^7 mL^{-1} in most studied ecosystems, and are known to continuously regulate microbial ecology and activity by affecting carbon and nutrient fluxes, food web dynamics and microbial diversity and diversification (Suttle, 2007; Shapiro and Kushmaro, 2011). Whilst viruses, including bacteriophages, are known to be found at high abundance and diversity in AS (10^8–10^9 virus like particles (VLP)/mL), they have proven difficult and time consuming to study (Otawa

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et al., 2007; Tamaki et al., 2012). Consequently our knowledge and understanding of phage ecology in AS systems, and their potential influence on these globally important processes, is limited.

Traditionally, viruses have been enumerated by culture based methods (Adams, 1959; Havelaar and Hogeboom, 1983; Kott, 1966) or by transmission electron microscopy (TEM) (Torrella and Morita, 1979; Bergh et al., 1989). The former is selective for host-specific infectious viruses, thus counts only represent a small fraction of the total population. Whilst the latter, though providing information on phage shape and size, is expensive, time consuming and lacks precision (Weinbauer, 2004). Over the past two decades the introduction of highly sensitive fluorescent nucleic acid-specific dyes (for example SYBR Green I, DAPI, and YOPRO-1) in combination with epi-fluorescence microscopy (EFM) has significantly improved the detection and quantification of viruses in aquatic ecosystems (Brussaard, 2004; Brussaard et al., 2010). EFM is considerably quicker, incurs lower costs and thus allows for a greater throughput of samples compared with TEM. With the introduction of flow cytometric detection and enumeration of free viruses, again in combination with sensitive nucleic acid-specific dyes, the sensitivity of detection, accuracy and precision of quantification and the speed of analysis has further improved. Consequently flow cytometry (FCM) has become the method of choice for quantifying viruses in aquatic samples (Brussaard et al., 2010). Despite this, virus abundance in AS has only been determined using TEM or EFM and not FCM, though the literature in this area is still modest (Ewert and Paynter, 1980; Otawa et al., 2007; Wu and Liu, 2009).

The aim of this paper is to critically describe a rapid FCM protocol to enumerate planktonic and floc-associated extracellular viruses in AS, to evaluate the protocol against that of Brussaard et al. (2010) and a TEM based approach, and to present virus abundance data from 25 AS plants.

2. Materials and methods

2.1. Protocol optimisation

2.1.1. Samples

AS samples were collected from a nitrifying domestic wastewater treatment plant (WWTP) in Tudhoe Mill, Durham, United Kingdom (UK), in March 2013. Samples were collected in polypropylene containers, stored at ~4 °C during transit and fixed within 2 h, as previously reported by Brussaard et al. (2010). Briefly 1 mL aliquots of each sample were transferred to 2 mL cryovials and fixed at a final concentration of 0.5% Glutaraldehyde for 15–30 min at 4 °C in the dark. After fixation aliquots were flash frozen in liquid nitrogen and stored at −80 °C. Samples were thawed at room temperature and mixed via manual shaking for 10 s prior to pre-treatment. Once established optimal pre-treatments were used in subsequent experiments.

2.1.2. Pre-treatments for dislodgment of floc bound viruses

2.1.2.1. *Chemical treatment*. Four dispersants, the surfactants – polyoxyethylene-sorbitan monooleate (Tween 80, Sigma) and Triton X-100 (TX, Sigma), and the ionic dispersants – sodium pyrophosphate (SP, Sigma) and sodium cholate (SC, Sigma), were tested separately and in combination at various concentrations as a sample pre-treatment for virus dislodgment from AS flocs (1 and 5% for Tween 80 and TX, 5 and 10 mM for SP and 0.1 and 1% for SC). Thus once thawed the dispersants were added to samples and incubated for 15 min in the dark at room temperature. All dispersants, with the exception of Tween 80, were autoclaved prior to use. Each treatment was analysed in triplicate, with a paired control (dispersant free samples) per replicate.

2.1.2.2. *Physical treatment*. The effect of ultrasound treatment, in combination with chemical treatment, on virus dislodgment was tested using a sonicating water bath (Decon FS200b; 120 W; 40 KHz), with 1 mL samples being run for 1, 2, 3, 5 and 8 min. Sonication was interrupted for 30 s every minute, during which time the samples were shaken manually (Danovaro et al., 2001). Each treatment was analysed in triplicate, with a paired control (samples without sonication) per replicate.

2.1.3. Extracellular DNA interference

In order to eliminate the uncertainties in virus counting due to extracellular DNA (eDNA) a nuclease treatment was tested, since viral nucleic acids will generally be protected from DNase degradation by their protein capsids and sometimes by a lipid envelope (Allander et al., 2001; Breitbart and Rohwer, 2005). DNase I (Qiagen, UK), at concentrations of 1500 U/μL and 1.5 U/μL, was added to samples and incubated for 15 min in the dark at room temperature. Each treatment, in addition to a DNase free sample (control), was analysed in triplicate.

2.1.4. Staining optimisation

SYBR Green I (SG I), SYBR Green II (SG II) and SYBR Gold (SG), which are used to stain double stranded DNA (dsDNA), single stranded DNA (ssDNA) and RNA and dsDNA, ssDNA and RNA respectively, were tested separately, to count specific virus communities, and in combination, to achieve the greatest total count, at various dilutions (0.5×10^{-4} and 1×10^{-4} of each stains stock solution respectively). To further optimise the staining procedure incubation temperatures of 75, 80 and 85 °C were also investigated. All stain combinations and incubation temperatures were analysed in triplicate.

2.2. Fluorescent staining and FCM analysis

AS samples were diluted with TE-buffer (10 mM Tris–HCl 1 mM EDTA; pH 8.0) to achieve an event rate between 200 and 800 viruses s^{-1} and avoid coincidence (i.e., two or more viruses and/or particles being simultaneously within the sensing zone). To achieve this five 1 mL dilutions (1/500, 1/750, 1/1000, 1/1250 and 1/1500) were prepared per replicate. Diluted samples were then stained using either the protocol of Brussaard et al. (2010), 10 μL of 0.02 μm filtered SYBR Green I (0.5×10^{-4} dilution of the commercial stock) for 10 min in the dark at 80 °C, or variations of this regarding staining optimisation. Sample dilutions were analysed in triplicate using a FACScan flow cytometer (Becton Dickinson, California) equipped with a 15-mW 488-nm air-cooled argon-ion laser and a standard filter setup. The trigger was set on green fluorescence (GFL).

Table 1 – WWTP details, AS process configurations and sample dates.

WWTP	Process Configuration	Aeration	Wastewater type	TEP[a]	Sampling date
Amble	SBR	Fine bubble	Municipal	16607	02.05.13
Aycliffe	Conventional	Jet	Municipal/Industrial	61106	04.04.13
Berwick	Conventional	Surface	Municipal	15537	21.05.13
Billingham	Conventional	Fine bubble	Municipal	35293	15.05.13
Blyth	SBR	Fine bubble	Municipal	37859	13.05.13
Bowsden	Oxidation Ditch	Surface	Municipal	250	21.05.13
Bran Sands	Conventional	Jet	Municipal/Industrial	391142	20.05.13
Branxton	Oxidation Ditch	Surface	Municipal	250	21.05.13
Broomhaugh	Oxidation Ditch	Surface	Municipal	7095	11.04.13
Browney	Conventional	Jet	Municipal	21586	24.05.13
Cambois	Conventional	Fine bubble	Municipal	28655	13.05.13
Cramlington	Conventional	Surface	Municipal/Industrial	45309	05.06.13
Haggerston	Oxidation Ditch	Medium bubble	Municipal	2040	21.05.13
Hendon	Conventional	Fine bubble	Municipal	229108	09.04.13
Hexham	Conventional	Surface	Municipal	29714	11.04.13
Hordon	Conventional	Fine bubble	Municipal	100299	09.04.13
Howdon	Oxidation Ditch	Fine bubble	Municipal/Industrial	947811	13.04.13
Marske	SBR	Fine bubble	Municipal	93556	09.04.13
Newbiggin	Conventional	Fine bubble	Municipal	38487	13.05.13
Seaham	Conventional	Fine bubble	Municipal	23595	15.05.13
Seahouses	SBR	Jet	Municipal	11213	02.05.13
Seaton Carew	Conventional	Fine bubble	Municipal	120222	09.04.13
Sedgeletch	Conventional	Fine bubble	Municipal	51152	04.04.13
Tudhoe Mill	Conventional	Fine bubble	Municipal	22493	30.04.13
Washington	Oxidation Ditch	Surface	Municipal/Industrial	74916	09.05.13

[a] Total equivalent population (TEP) served by the plant. SBR: Sequencing batch reactor.

Highly diluted and well-mixed yellow-green fluorescent microspheres (FluoSpheres, 1.0 μm diameter; Invitrogen, Molecular Probes; F8823) were added as an internal reference to all samples. Readings were collected in logarithmic mode (at least 5000 events per sample) and analysed with FlowJo v10.0.7r2 (FlowJo LLC, Oregon). Data was collected using GFL/side scatter (SSC) dot plots and specified gates taken from Brussaard et al. (2010), V1, V2 and V3 which correspond to viruses of differing fluorescence intensity (total count = V1+V2+V3). This enabled optimal distinction between stained viruses and other microbial cells and/or background noise, thus filtration to remove such particles wasn't required. Blanks, consisting of TE-buffer and autoclaved 0.2-μm-filtered sample, were pre-treated and analysed identically to samples, further facilitating the correction of virus counts for noise.

2.3. Virus recovery efficiency

AS samples, collected from Tudhoe Mill WWTP, were seeded with the dsDNA coliphage T4 (NCIMB, UK) and left for 15 min prior to fixing. Triplicate samples, with and without the seeded T4 coliphage, were then prepared and analysed following both the optimised protocol and that of Brussaard et al. (2010). The seeded T4 abundance was determined by FCM ($0.91 \pm 0.04 \times 10^9$) and, for comparison, by plaque assay ($1.0 \pm 0.17 \times 10^9$). Briefly 20 μL of the host isolate *Escherichia coli* was suspended in 8 mL of sterile sloppy agar (0.5% agar in nutrient broth medium) together with 20 μL of filter-fertilised (0.2 μm) T4 coliphage culture. The sloppy agar was then poured over a pre-warmed (37 °C) nutrient agar plate and incubated for 2 days at 37 °C. Plates were checked after 24 and 48 h for plaque formation. The FCM seeded concentration of 0.91×10^9 was used for calculations.

2.4. Virus abundance at a suite of AS WWTP's

AS samples were collected from 25 domestic WWTP's situated within the North East of England, UK, in April and May 2013 (see Table 1 for plant configurations/characteristics). Triplicate samples were collected, fixed and then analysed using the optimised protocol (Fig. 7). The mixed liquor (volatile) suspended solids (MLSS/MLVSS) were determined according to Standard Methods (APHA, 1998).

2.5. Comparison of FCM and TEM counts

FCM AS viral counts obtained from 7 of the WWTP's were compared with TEM counts. For TEM analyses the preconcentration procedure (i.e. ultracentrifugation) typically used was omitted, since the number of viruses in AS was expected to be very high (Otawa et al., 2007; Wu and Liu, 2009). 1 mL of pre-treated sample was diluted with 1 mL of deionised water, mixed and then 2 μL was spotted onto a 200 mesh Formvar coated copper grid and air dried at room temperature. Unrinsed grids were negatively stained with 2% uranyl acetate for 1 min. Excess stain was drained off with a pointed piece of glass fibre filter paper and grids were then left to dry at room temperature for 24 h. Observations were made using a Philips CM 100 compustage transmission electron microscope, operating at 100 kV (Fig. 6b). Duplicate grids were prepared for each sample, with 30 fields of view (FOV), determined as a sufficient sample size as described by Davenport and Curtis (2004), examined per grid at a magnification of 13,500.

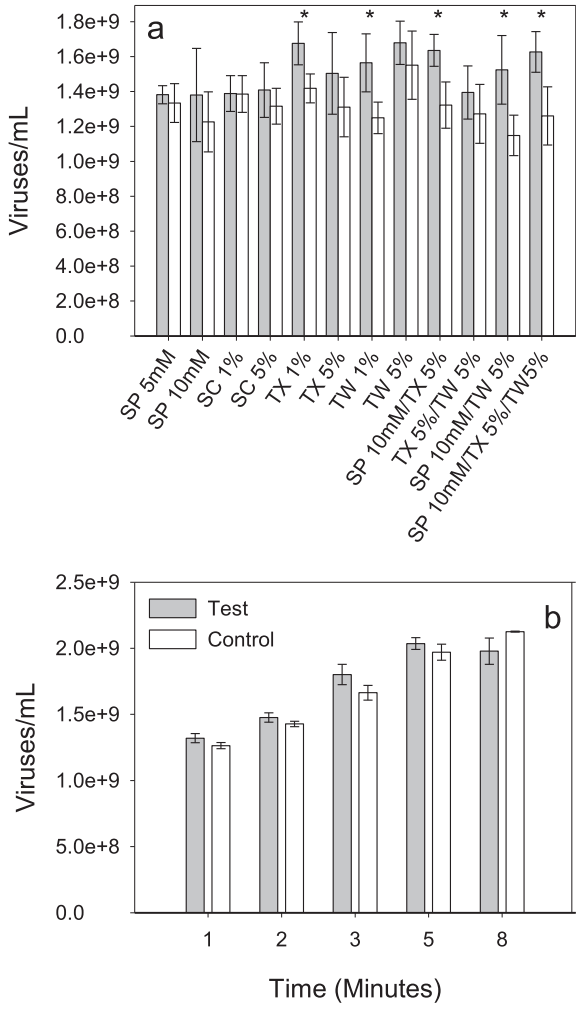

Fig. 1 – Effect of dispersants (a) and sonication time (b) on the dislodgment of indigenous viruses from AS. Main bars indicate mean virus abundance, while the error bars indicate the standard deviation of three replicates. The treatments found to significantly affect total virus counts are indicated by an asterisks: *, $P < 0.05$.

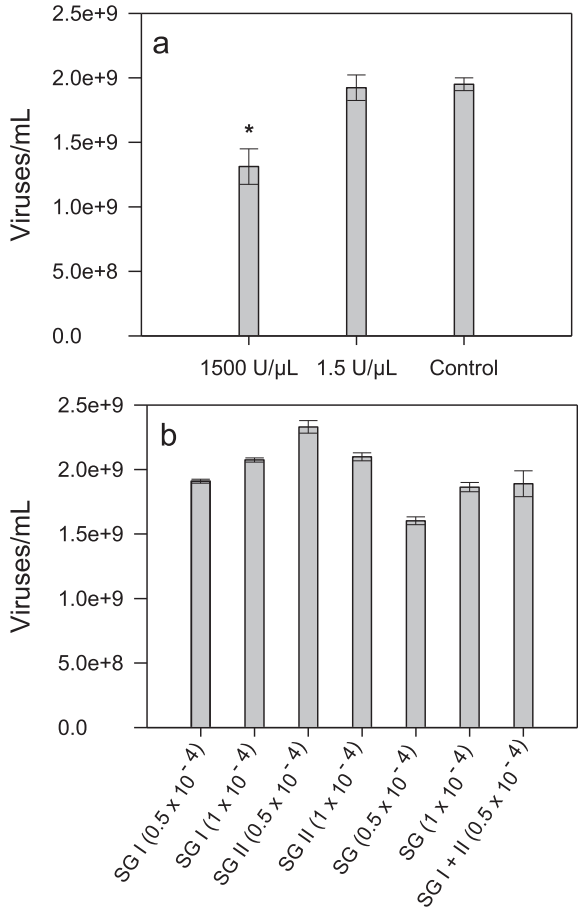

Fig. 2 – Effect of DNase treatment (a) and stain type and dilution (b) on counts of indigenous viruses from AS. Bars indicate mean virus abundance, while the error bars indicate the standard deviation of three replicates. The treatments found to significantly affect total virus counts are indicated by asterisks:*, $P < 0.05$.

2.6. *Statistical analyses*

Virus concentrations found after each treatment were compared and analysed for significance using ANOVA with Tukey's pairwise comparisons. Its use was justified as the data was normally distributed and showed homoscedasticity. A Pearson product–moment correlation coefficient was calculated to determine the relationship between FCM and TEM counts. All statistical analysis was performed in Minitab v16.1.0.

3. Results

3.1. *Optimisation of protocol for AS virus enumeration by FCM*

AS samples incubated with dispersants displayed higher virus counts than untreated samples (Fig. 1a). The most effective treatment, based on the largest increase in virus abundance from its paired control, was Tween 80 (5%) and SP (10 mM); $1.52 \pm 0.19 \times 10^9$ treated and $1.15 \pm 0.12 \times 10^9$ control (ANOVA: $p = <0.05$).

Sonication had no statistically significant effect in four pairwise comparisons (1, 2, 3 and 5 min) with unsonicated samples (ANOVA: $p = >0.05$). 8 min had a non-significant negative effect on virus counts (ANOVA: $p = >0.05$) (Fig. 1b).

Virus counts obtained from DNase treated samples gave contrasting results (Fig. 2a). Samples treated with 1500U/µL gave significantly lower counts than those in untreated samples ($1.31 \pm 0.14 \times 10^9$ and $1.95 \pm 0.05 \times 10^9$ respectively, ANOVA: $p = <0.05$), a percentage decrease of 32.7%, whilst samples treated with 1.5U/µL showed no significant difference to those in untreated samples ($1.92 \pm 0.1 \times 10^9$ and $1.95 \pm 0.05 \times 10^9$ respectively, ANOVA: $p = >0.05$) (Fig. 2a).

The highest virus count was achieved using SG II at a dilution of 0.5×10^{-4} ($2.3 \pm 0.05 \times 10^9$) (Fig. 2b), although counts were not significantly higher than those obtained using SG I, SG or SG I + II (ANOVA: $p = >0.05$). No large difference in GFL or SSC single was detected between the three stains, thus distinguishing

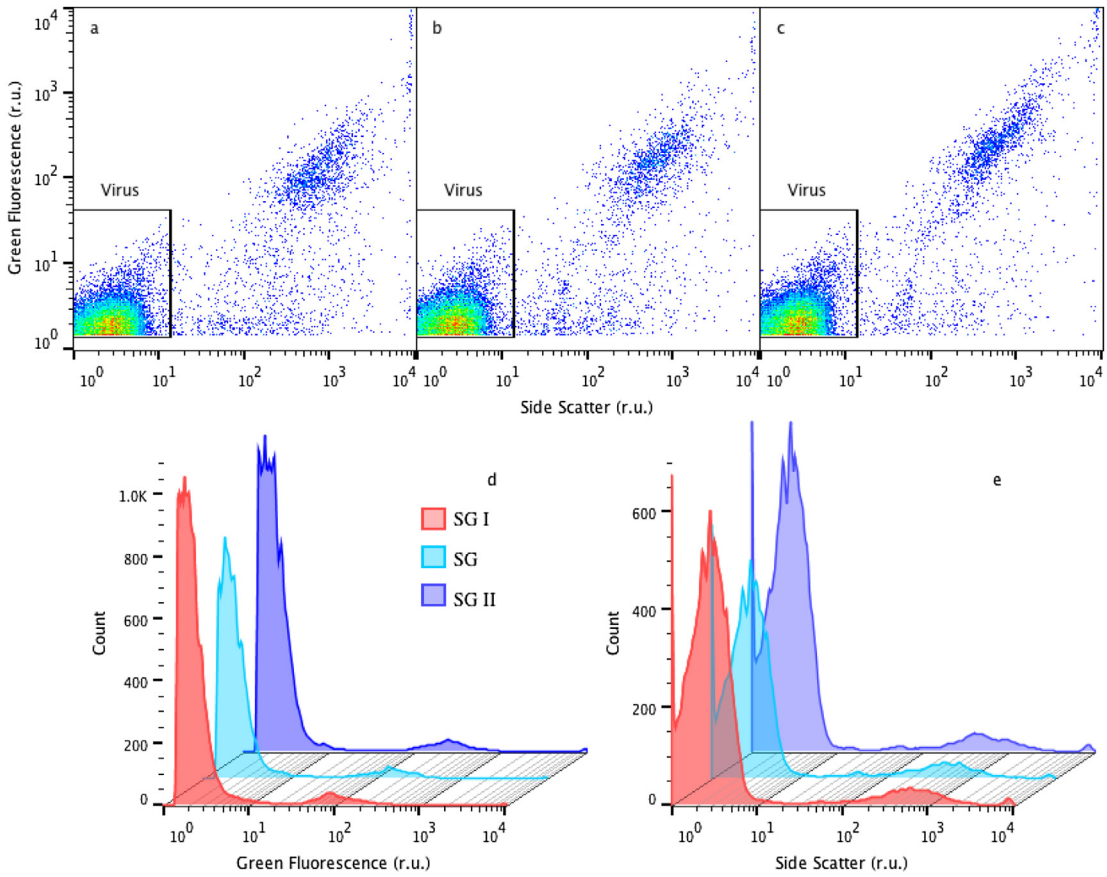

Fig. 3 – Cytograms (a–c) and historgrams (d–f) of AS samples taken from Tudhoe Mill WWTP stained with SG I (a), SG (b) and SG II (c), all 0.5 × 10^{-4} dilutions. All events plotted, 1/1000 dilution. r.u. relative units. Total Count (virus) = V1+V2+V3, gates are from Brussaard et al. (2010).

between dsDNA, ssDNA or RNA viruses was not possible (Fig. 3). The original incubation temperature of 80 °C gave the highest counts (1.97 ± 0.02 × 10^9), they were not however significantly greater than those obtained at 75 and 85 °C (1.82 ± 0.01 × 10^9 and 1.87 ± 0.03 × 10^9, ANOVA: p = >0.05) respectively (Fig. 4).

3.2. *Virus recovery and enumeration efficiency*

The efficiency of virus detachment and staining for both protocols was tested by estimating the recovery of the T4 virus from seeded samples, as well as total virus recovery. The recovery efficiency of the seeded T4 coliphage varied between the two protocols, with the optimised protocol presented here recovering 102 ± 2.7% compared to that of Brussaard et al. (2010), which recovered 85.4 ± 2.1% (0.93 ± 0.02 × 10^9 mL^{-1} and 0.78 ± 0.02 × 10^9 mL^{-1} of the 0.91 × 10^9 seeded abundance respectively). Total virus recovery also varied, the optimised protocol recovered 1.07 ± 0.03 × 10^9 mL^{-1} compared with 0.87 ± 0.02 × 10^9 mL^{-1} recovered by that of Brussaard et al. (2010), an increase of 22.9%.

3.3. *Virus abundance in full scale activated sludge WWTP's*

Virus abundance in 25 AS plants ranged from 0.59 ± 0.04 × 10^9 mL^{-1} (Bowsden) to 5.14 ± 0.37 × 10^9 mL^{-1} (Howdon), with a mean concentration of 2.35 × 10^9 mL^{-1} (Table 2). The concentration of viruses per gram (dry) of MLSS ranged from 2.64 ± 0.10 × 10^{11} (Brand Sands) to 28.11 ± 3.15 × 10^{11} (Washington), with a mean concentration of 9.59 × 10^{11}. 93.8% ± 2.4% of viruses found across all plants were those associated with the V1 subpopulation, with the V2 and V3 subpopulations making up 6.3 ± 2.5% and 0.3 ± 0.1% respectively (Fig. 5). No clear relationship was apparent between MLSS and virus concentrations.

3.4. *FCM vs. TEM*

The direct comparison of FCM and TEM viral counts were highly correlated (p = <0.05 and r^2 = 0.77), suggesting FCM is suitable for enumeration of viruses in AS (Fig. 6a). However FCM estimates of virus abundance were always higher than those given by TEM, by an average factor of 2.7. The reproducibility of the FCM method is much greater, with the average coefficient of variance between replicates being 6.67 for FCM and 17.25 for TEM, a factor of 2.58.

4. Discussion

We present a rapid, accurate and sensitive flow cytometric method specifically optimised for enumerating total

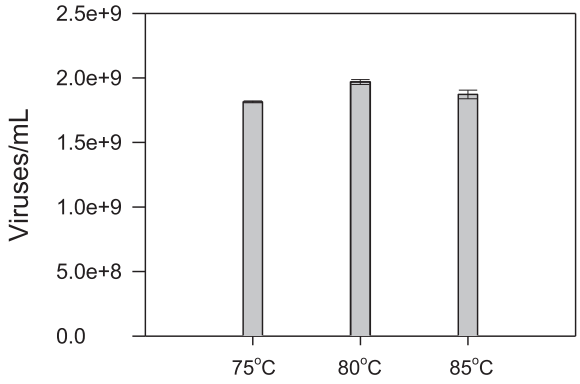

Fig. 4 – Effect of incubation temperature on virus counts from AS. Bars indicate mean virus abundance, while the error bars indicate the standard deviation of three replicates.

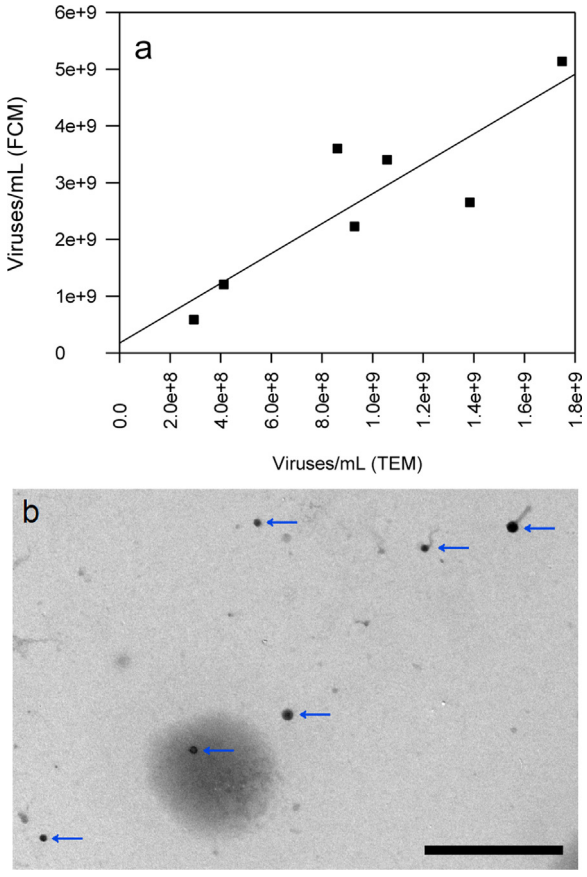

Fig. 6 – Relationship between AS virus counts enumerated by FCM and TEM (a) and a transmission electron micrograph (b) showing a typical counting FOV. Bar represents 1 μm. Blue arrows indicate virus particles.

planktonic and floc-associated extracellular viruses in AS. This constitutes an improvement in the study of AS viral communities because FCM is faster and less dependent on the operator than EFM and TEM. The performance of FCM virus quantification, is however strongly affected by AS sample pre-treatment, optimisation of the staining procedure and the presence of false positives, i.e. the staining of DNA associated with membrane-derived vesicles (MVs), gene transfer agents (GTAs) and eDNA (Forterre et al., 2013).

The very different effects of sample pre-treatment emphasises the importance of selecting appropriate techniques to enable accurate virus quantification in AS samples. Dispersants have previously been used successfully as an eluent for dislodging viruses from sludge (Wu and Liu, 2009) and marine (Danovaro et al., 2001; Danovaro and Middelboe, 2010) and freshwater (Duhamel and Jacquet, 2006) sediments. This survey confirms these findings: the addition of SP (10 mM) in combination with Tween 80 (5%) producing the highest and most accurate counts. Sonication has also been used to dislodge viruses from marine (Danovaro et al., 2001; Danovaro and Middelboe, 2010) and freshwater sediments (Duhamel and Jacquet, 2006), soils (Williamson et al., 2003) and anaerobic digester sludge (Wu and Liu, 2009), with optimum sonication times of 30 s (Otawa et al., 2007) and 1 min (Wu and Liu, 2009) reported for AS samples. No significant effect was observed in this study. Possible explanations could be the more powerful equipment (120 W compared to 10 W and

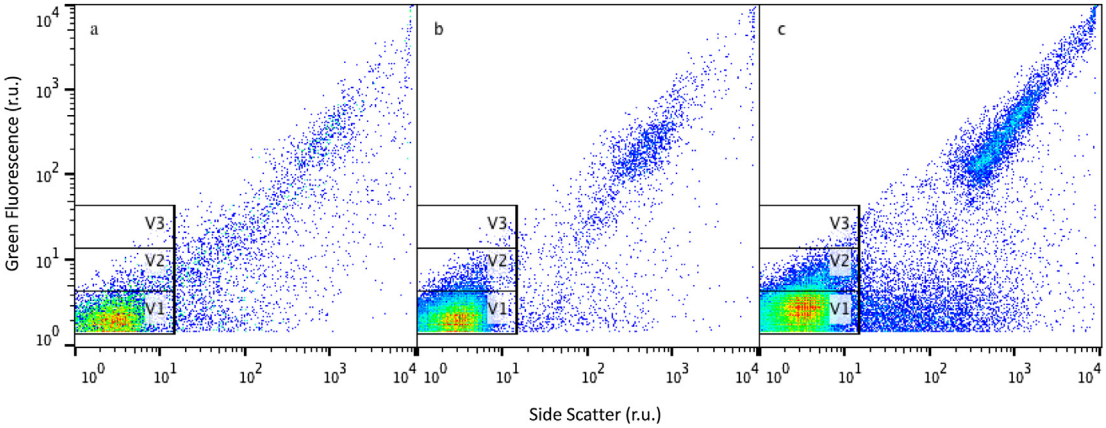

Fig. 5 – Cytograms of AS samples taken from Bowsden (a), Sea Houses (b) and Howdon (c) WWTP's. All events plotted, 1/1000 dilution. r.u. relative units. V1, V2 and V3 gates are taken from Brussaard et al. (2010). Total Count = V1+V2+V3.

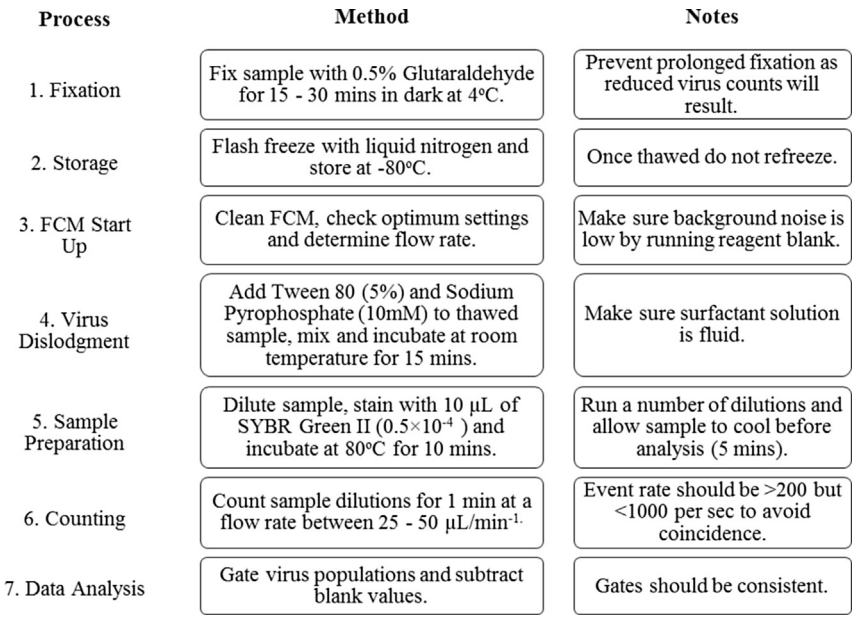

Fig. 7 – Diagram of the optimised protocol suggested on the basis of our findings, including processes, methodology and critical notes.

Table 2 – Concentration of viruses from 25 activated sludge plants in the North East of England, UK.

WWTP	Virus concentration[a] (10^9/mL^{-1})	MLSS (g/L)	Virus concentration[b] (10^{11} g^{-1})
Amble	3.25 ± 0.16	3.1	10.48 ± 0.51
Aycliffe	1.81 ± 0.15	1.9	9.51 ± 0.79
Berwick	1.21 ± 0.09	2.09	5.78 ± 0.43
Billingham	1.89 ± 0.10	1.36	13.92 ± 0.73
Blyth	3.40 ± 0.18	4.58	7.43 ± 0.39
Bowsden	0.59 ± 0.04	2.13	2.76 ± 0.21
Bran Sands	2.96 ± 0.11	11.21	2.64 ± 0.10
Branxton	1.05 ± 0.07	2.02	5.21 ± 0.34
Broomhaugh	1.35 ± 0.15	4.01	3.38 ± 0.39
Browney	0.72 ± 0.08	1.87	3.83 ± 0.41
Cambois	2.23 ± 0.07	2.88	7.75 ± 0.23
Cramlington	3.54 ± 0.23	1.38	25.65 ± 1.66
Haggerston	1.23 ± 0.05	2.86	4.30 ± 0.19
Hendon	3.25 ± 0.13	3.08	10.55 ± 0.44
Hexham	2.51 ± 0.13	2.77	9.05 ± 0.45
Hordon	2.23 ± 0.21	2.16	10.33 ± 0.97
Howdon	5.14 ± 0.37	2.19	23.46 ± 1.71
Marske	3.60 ± 0.21	2.94	12.25 ± 0.71
Newbiggin	2.88 ± 0.34	4.23	6.82 ± 0.79
Seaham	1.00 ± 0.04	1.54	6.54 ± 0.25
Seahouses	2.41 ± 0.19	3.22	7.49 ± 0.59
Seaton Carew	2.65 ± 0.11	3.1	8.56 ± 0.35
Sedgeletch	1.12 ± 0.06	3.04	3.68 ± 0.21
Tudhoe Mill	2.70 ± 0.47	2.64	10.21 ± 1.79
Washington	3.98 ± 0.45	1.415	11.11 ± 3.15

[a] Concentrations determined by FCM using the optimised protocol, ± denotes standard deviation between triplicate samples.
[b] Values calculated from virus concentration per millilitre and the MLSS data.

100 W), smaller sample size (1 mL compared to 10 mL and 50 mL) or greater dislodgment efficiency of the chemical pre-treatment used in this study. Disruption of viral protein capsids or lipid envelopes is thought to occur with enhanced sonication times (Wu and Liu, 2009) and may have lowered counts in this study.

FCM enumeration of free viruses requires working close to the limits of staining methodology and the detection limit of a flow cytometer, thus the intensity of the GFL and/or SSC signal is crucial for optimising such protocols. SG I is commonly used for counting pelagic marine viruses (Marie et al., 1999; Brussaard, 2004) by FCM, however in some instances SG and SG II have provided increased and more reproducible counts (Chen et al., 2001; Duhamel and Jacquet, 2006; Tomaru and Nagasaki, 2007). Our results suggest SG II at a dilution of 0.5×10^{-4} provides the most accurate enumeration of total free viruses in AS. SG II has a strong affinity to RNA and thus a greater ability, when compared to SG I and SG, to stain small genome sized RNA viruses, which could explain the small increase in counts recorded. However the total counts and GFL/SSC signals observed would imply that all three dyes have a very similar ability to stain dsDNA, ssDNA and RNA viruses respectively, a finding also reported by Brussaard et al. (2000) and Brussaard (2004). Consequently distinguishing between these virus populations is not possible with the method presented, as it is apparent that the total count obtained encompasses all three.

Another important factor when trying to increase GFL is the incubation temperature, as heat treatment affects the permeability of the viral capsid and denatures the nucleic acid, thereby improving staining efficiency (Brussaard, 2004). An incubation temperature of 80 °C is most commonly used for FCM enumeration of pelagic marine viruses (Marie et al., 1999; Brussaard, 2004), however incubation at room

temperature and 75 °C has been shown to provide increased and more reproducible counts in marine samples (Tomaru and Nagasaki, 2007) and freshwater sediments (Duhamel and Jacquet, 2006). Our results suggest an incubation temperature of 80 °C provide the most accurate enumeration of AS viruses.

The significant ($p = <0.05$) relationship between FCM and TEM counts obtained using the linear regression model has a correlation coefficient of 0.77 and a non-significant intercept, suggesting that these two methods are evaluating the same virus particles. However the FCM counts were typically 2.7 times higher than corresponding TEM values. A direct comparison of FCM and TEM has never previously been undertaken. However direct comparisons of EFM and TEM for marine and freshwater environments suggest a similar discrepancy between the fluorescent and direct counts with Hennes and Suttle (1995), Weinbauer and Suttle (1997) and Noble and Fuhrman (1998) reporting differentials of 2.3, 1.5 and 1.3 respectively.

Discrepancies could result from the presence of false positives, eDNA, GTAs and MVs, causing FCM to overestimate virus abundance, a growing concern in natural environments (Forterre et al., 2013). Treatment with DNase has previously been used to eliminate or reduce such an outcome, although no significant difference in EFM virus counts was observed by Otawa et al. (2007) and Wu and Liu (2009) between treated and untreated AS samples. Our results gave contrasting results, with the more concentrated DNase samples showing a significant decline in virus counts and the less concentrated samples showing little affect. The sensitivity of viruses to DNase has been demonstrated previously (Jiang and Paul, 1995; Bettarel et al., 2000), it is probable that at the higher DNase concentrations true viruses were degraded and thus counts reduced. MVs produced by Proteobacteria, which dominate AS communities (Wagner et al., 2002), and some hyperthermophilic archaea, as well eDNA adsorbed to cell debris or mineral surfaces, are also known to produce false EFM and FCM positives even after DNase treatment (Nielsen et al., 2007; Soler et al., 2008; Zhao et al., 2010). As there are no good methods to rapidly discriminate between viruses, GTA'S, MV'S and eDNA and DNase is ineffective in their removal and can degrade the viruses of interest, it is recommended that an FCM (or EFM) count be controlled for the presence or absence of such false positives by a TEM count in a selection of samples, as done in this study.

Discrepancies might also be caused by particulate matter and detritus obscuring the virus particles during TEM counts (Hennes et al., 1995; Betteral et al., 2000). It was difficult in this study to find areas of the TEM grids devoid of such particles, though sufficient clear FOV were found to make an accurate count. The underestimation of values and significant greater variability of the TEM method in comparison to FCM may also be explained by the high magnifications used and the potential loss of viruses during the staining procedure (Betteral et al., 2000).

The abundance of viruses in AS, as determined from 25 WWTP's, is of the order 10^8–10^9 mL^{-1}, similar to results reported by Otawa et al. (2007) and Wu and Liu (2009) (2.35×10^9 mL^{-1} compared with 1.1×10^9 and 1.19×10^9 mL^{-1} respectively). The concentration of viruses per gram (dry) of MLSS was also within the same order of magnitude, 10^{11}–10^{12} g^{-1}, across all three studies. The majority of viruses found were those associated with the low and medium fluorescence intensity V1 and V2 virus subpopulations, thought to be bacteriophages of the smallest class (30–60 nm in size) (Marie et al., 1999; Brussaard et al., 2010). Whilst V1 viruses are thought to be smaller in size than V2 viruses, true size estimates are not viable since the GFL and SSC signals are not related to genome size or virus size or shape (Marie et al., 1999; Brussaard, 2000).

The concentration of viruses in AS is thus amongst the highest of all systems studied to date. In marine environments concentrations range from 10^4 and 10^8 mL^{-1} (Wommack and Colwell, 2000), in freshwater ecosystems the highest virus abundance to date is 9.6×10^9 mL^{-1} (Hennes et al., 1995) whilst in marine and freshwater sediments virus abundance ranges from $0.03–11.7 \times 10^9$ g^{-1} (Danovaro et al., 2002). In such environments viruses are proposed to continuously regulate microbial activity and ecology, including carbon and nutrient fluxes, food web dynamics and microbial diversity and diversification (Weinbauer, 2004). Given the high concentrations found in this study and the apparent dominance of bacteriophages it is speculated that viruses are active and dynamic in AS processes and could, in theory, influence microbial activity and ecology, thus directly affecting system performance and functional stability. The availability of a rapid quantification method will facilitate in testing this hypothesis.

5. Conclusions

- The results show that the optimised protocol presented is an accurate and highly reproducible method for enumerating total free viruses in AS and thus is ideal for routine investigation.
- FCM counts were highly correlated with TEM based counts and results were comparable to previously published EFM counts.
- The major advantage of FCM over TEM and EFM is its high throughput, removing a key obstacle to undertaking detailed spatial and temporal studies of virus dynamics in AS systems. Such studies are a fundamental prerequisite to understanding their possible impact on a systems bacterial population and thus performance and functional stability.

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